

Soluble mesothelin related peptides (SMRP) and osteopontin as protein biomarkers for malignant mesothelioma: analytical validation of ELISA based assays and characterization at mRNA and protein levels

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Abstract

Background: There is a need to identify reliable markers for malignant mesothelioma. Soluble mesothelin related peptides (SMRP) and osteopontin (OPN) have gained interest in recent years for this purpose.

Methods: SMRP (Fujirebio Diagnostics Inc.) and OPN (R&D Inc.) ELISA methods were evaluated for multiple parameters. Concentrations were measured in blood from patients with mesothelioma, normal healthy volunteers, and patients with other (non-mesothelioma) cancers. In silico analysis was performed using the GeoProfiles database. At the protein level, SMRP and OPN were measured in cell culture supernatants, and values were compared in patients pre- and post-extrapleural pneumonectomy.

Results: The SMRP assay demonstrates intra-assay CVs of 2.3% and 3% (at 4.6 nM and 13.7 nM, respectively), and inter-assay CVs of 3.5% and 3.7% at the same concentrations. The limit of detection (LOD) is 0.182 nM. The OPN assay demonstrates intra-assay CVs of 5.8%, 4.1%, and 5.2% (at 1.9, 5.1, and 11.1 ng/mL, respectively), and inter-assay CVs of 8.5%, 8.4%, and 12.1% at the same concentrations. The LOD is 0.032 ng/mL. Both SMRP and OPN in mesothelioma patients were significantly higher than in patients with other (non-mesothelioma) cancer and in healthy volunteers. The two markers do not appear to correlate with each other and exhibit different tissue expression patterns.

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Received February 24, 2009; accepted November 1, 2009

Protein concentrations of these markers are highest in different sets of cell lines. Finally, SMRP but not OPN concentrations were decreased in five of seven consecutive patients after extrapleural pneumonectomy, compared to their respective pre-operative values.

Conclusions: These assays provide reliable and reproducible quantitation of SMRP and OPN proteins. Both are increased in mesothelioma patients compared to non-mesothelioma controls. However, the two analytes do not correlate with each other and show distinct expression profiles and protein expression. Concentrations of SMRP but not OPN are decreased in post-surgical samples. Our results further characterize these markers, establish assay performance characteristics, and lay the groundwork for further studies to measure these markers in blood.

Clin Chem Lab Med 2010;48:271–8.

Keywords: analytical validation; cancer biomarkers; mesothelin; mesothelioma; osteopontin; soluble mesothelin related peptides.

Introduction

Diffuse malignant pleural mesothelioma is an uncommon and lethal cancer for which there is currently no universally accepted treatment standard (1–4). The incidence of malignant mesothelioma is estimated at 2000–3000 cases annually in the USA (5). In Western Europe alone, a quarter of a million deaths are projected over the next 30 years (6). It is important for thoracic physicians to be knowledgeable about mesothelioma because they are often required to make a diagnosis and recommend treatment.

Diagnosis of mesothelioma can be difficult. It presents with a wide range of morphological characteristics on histology, making it difficult to differentiate from other malignancies (7). Similarities exist between epithelioid mesothelioma and metastatic adenocarcinoma. Immunohistochemistry has been useful in differentiating mesothelioma from adenocarcinoma. Many have found the distinction by identifying calretinin in mesothelioma and carcinoembryonic antigen (CEA) in adenocarcinoma. Other difficulties exist in differentiating sarcomatoid mesothelioma from synovial sarcoma, desmoplastic mesothelioma from pleural fibrosis, and well-differentiated mesothelioma from papillary hyperplasia. No marker has 100% sensitivity or specificity for the diagnosis of mesothelioma, and commonly a panel of markers,

such as CEA, tumor-associated glycoprotein 72 [B72.3], Leu-M1, vimentin, thrombomodulin, secretory component, carcinoma antigen-125, and mucin is employed (8). Sometimes the distinction is still not possible and electron microscopy is used to identify the numerous long microvilli present in mesothelioma. A biomarker specific for the diagnosis of mesothelioma would prove very useful for accurate diagnosis.

Malignant pleural mesothelioma is also difficult to evaluate radiologically due to its propensity to infiltrate locally into and along tissue planes. Computed tomography and magnetic resonance imaging have been used to clinically stage disease. Unfortunately, there are major discrepancies when compared to surgical–pathological findings (9, 10). There is evidence that the treatment of early stage disease using extrapleural pneumonectomy or pleurectomy/decortication may improve survival (11–14). However, ~20%–30% of patients undergo exploratory thoracotomy without resection because of the unreliability of current radiological modalities in predicting locally advanced disease (i.e., unresectable disease) (2). Several studies have suggested that FDG PET (position emission tomography) may be useful in the pre-operative staging of this disease (15, 16). A better method of determining extent of disease is necessary to improve surgical outcome and minimize the number of patients subjected to exploratory thoracotomy without surgical resection. A biomarker that is a surrogate marker for the extent of disease may help determine initial treatment, response to treatment, and determine recurrence of disease during routine follow-up.

Two markers, mesothelin and osteopontin (OPN), have garnered interest in recent years for monitoring therapy or detecting recurrence of mesothelioma. Mesothelin, encoded by the *MSLN* gene, is a member of the megakaryocyte potentiating factor (MPF) family and is produced specifically in mesothelium cells. It is believed to play a role in cellular adhesion. *MSLN* mRNA is translated into a preprotein form which is post-translationally cleaved into two products, MPF and mesothelin (17–19). Three mesothelin transcripts have been reported in the literature: variant 1 encoding MPF (NM_005823), variant 2 encoding mesothelin (NM_013404), and variant 3 (AF180951) (20–24). Studies from ovarian and pancreatic cancer cell lines and tissue support the existence of variants 1 and 3 only, with variant 1 being the predominant form (24). The mesothelin portion of this protein is detectable using an ELISA method which targets the soluble mesothelin related peptides (SMRP). Because mesothelioma is a rare disease, this assay has been FDA (Food and Drug Administration) approved for humanitarian use and its intended application is to aid in the monitoring of patients diagnosed with mesothelioma.

The second marker, OPN, is a glycoprotein that is abundantly expressed by several cancers including lung, breast, colorectal, gastric, ovarian, and melanoma (19). It is involved in cell adhesion and has been shown to distinguish mesothelioma patients from individuals that are exposed to asbestos without malignancy (25). There are three isoforms of this

protein present in human cells: NM_001040058, NM_000582, and NM_001040060 (26). OPN protein can be detected in the circulation using antibodies that target this protein.

The purpose of this study was to further characterize mesothelin and OPN. We performed an analytical and clinical validation of two ELISA based methods for these markers, established normal reference intervals for our patient population, and characterized the protein concentrations in blood samples from patients with different cancers. We also assessed both molecules at the mRNA level in human tissues and protein concentrations in cell line supernatants. Our results help characterize further both mRNA and proteins for these two markers, establish performance characteristics for these assays, and lay the groundwork for further studies.

Materials and methods

SMRP ELISA immunoassay

SMRP ELISA – Mesomark™ (Fujirebio Diagnostics Inc., Malvern, PA, USA) is a sandwich immunoassay used to quantitate SMRP in human serum. There is a six-point standard curve (0–32 nM) and the assay was performed per the manufacturer's instructions. All samples were measured in duplicate and the mean calculated. Briefly, 10 µL of serum samples to be tested are diluted into 1 mL of calibrator solution. 100 µL of calibrators, controls and diluted samples are dispensed into the appropriate well in the coated microtiter plate and then incubated on a shaker for 60 min. The plate is decanted and washed five times with 350 µL of wash buffer in each well. Subsequently, 100 µL of conjugate (monoclonal antibody specific for MSLN conjugated to horseradish peroxidase) is added and the plate incubated for 60 min. The plate is then washed five times with 350 µL of wash buffer in each well. 100 µL of substrate solution (tetramethylbenzidine) is added and the plate is incubated in the dark for 15 min. 1% hydrochloric acid is added to stop the reaction and the plate is read spectrophotometrically at 450 nm using a BioTek ELx808 ultra microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

Osteopontin immunoassay

The Quantikine® OPN immunoassay (R&D Systems Inc., Minneapolis, MN, USA) is a 4.5-h solid-phase ELISA that employs a sandwich immunoassay to quantitate human OPN in plasma (dilution required). The ELISA was performed per the manufacturer's instructions. All samples are measured in duplicate and the average calculated. Briefly, a standard curve (4-parameter fit) is generated by diluting a stock standard solution (200 ng/mL) to the following concentrations: 20, 10, 5, 2.5, 1.25, 0.625, 0.312, and 0 ng/mL. One hundred µL of diluent is added to 50 µL of standards, controls, or samples into designated wells of the microtiter plate, and coated with a monoclonal antibody specific to OPN. The plate is incubated at room temperature for 2 h. It is then washed four times with wash buffer and 200 µL of conjugate containing anti-OPN polyclonal antibody-horseradish peroxidase conjugate is added to the wells. After a 2-h, room temperature incubation, the plate is decanted and washed four times with 400 µL of wash buffer. Finally, 200 µL of substrate (tetramethylbenzidine) is added to the wells and the plate is incubated in the dark for 30 min. The reaction is stopped by the

addition of 50 μ L of 2N sulfuric acid and read at 450 nm with λ correction at 530 nm.

Patient recruitment

Institutional Review Board approval was obtained at Memorial Sloan-Kettering Cancer Center (MSK; New York, NY) for the collection of serum and heparinized plasma from study patients. Consecutive clinic patients for whom eligibility criteria were met, i.e., mesothelioma diagnosis without previous treatment, were used in this study. For these samples, the age range was 55–70 years, with a median of 64 years. The majority of mesothelioma samples were from patients in stage 2–3, including those that underwent extrapleural pneumonectomy. The remaining samples were from patients in stage 4. Greater than 90% of samples had an epithelioid component, i.e., comprised of pure epithelioid and also mixed histology specimens. Less than 10% of the remaining specimens were from patients with sarcomatoid mesothelioma.

Control samples were obtained from: 1) a pool of volunteer healthy donors without evidence of disease; these donors had an age range from 40 to 59 years, with a median of 54 years, 2) blood samples from patients ($n=10$ each group) diagnosed with prostate, breast, and ovarian cancer, most with late stage cancer, enrolled in clinical protocols at MSK.

All patients signed informed consent for collection of blood and tissue. A pilot cohort of seven consecutive patients who underwent extrapleural pneumonectomy had mesothelin and OPN concentrations measured before and after surgical resection at designated time intervals, with comparisons performed on 1 week pre-surgical and 6 month post-surgical samples.

Sample collection and processing

Blood samples were centrifuged and serum/plasma separated within 30 min of collection. All samples were spun at $1500 \times g$ for 10 min to separate cellular components. Serum or plasma were subsequently transferred to cryotubes and stored at -80°C , until further analysis.

Analytical parameters and statistical analysis

The limit of detection (LOD) was determined using 20 replicates of a blank solution and calculated as follows: $\text{LOD} = \text{mean} + 2 \text{ SD}$. Intra-assay precision was determined using 20 replicates of control samples at the appropriate concentrations, and the mean, SD, and CV (%) calculated. Inter-assay precision was calculated similarly, using four daily runs.

Receiver operating characteristic (ROC) curves were created using MedCalc (MedCalc Software, Mariakerke, Belgium) on a cohort of 201 samples (140 mesothelioma + non-mesothelioma).

For comparison of SMRP and OPN between different sample cohorts, a two-tailed heteroscedastic t-test was performed, with significance set at $p=0.05$. For comparison of pre-surgical and post-surgical samples, a paired, two-tailed Student's t-test was performed, and significance assessed using $p=0.05$.

Tissue survey mRNA

This tissue survey was performed *in silico* using the GEO profiles database (accession number: GDS1096/204885_s_at/MSLN/Homo sapiens for MSLN and GPL96/209875_s_at/Homo sapiens for OPN). Results show expression profiling of 36 types of normal tissue, with each RNA tissue sample pooled from several donors.

For additional experimental details, see reference (27). GAPDH mRNA was used for normalization.

Cell culture samples

Three mesothelioma cell lines (VAMT, Meso37, and HMeso), three lung cancer cell lines (A549, PC9, and H3255), and two other immortalized cell lines (HeLa cervical adenocarcinoma cells and HEK-293 human embryonal kidney cells) were used in this study. For the lung cancer cell lines, A549 cells have activating mutation in KRAS, PC9 cells have exon 19 deletion in epidermal growth factor receptor (EGFR) and are sensitive to EGFR inhibitors, and H3255 cells have the L858R mutation in EGFR and are sensitive to EGFR inhibitors. All are NSCLC adenocarcinomas. Cell lines were obtained courtesy of Molecular Diagnostics Service (Memorial Hospital of MSK, New York, NY). HeLa cells were grown in DMEM with 10% fetal calf serum (FCS) and penicillin (100 U/mL) and streptomycin (100 μ g/mL). The remaining cell lines were grown in RPMI-1640 with 10% FCS and penicillin (100 U/mL) and streptomycin (100 μ g/mL). All cells were grown in a humidified incubator at 37°C and 5% CO_2 .

On day 0 of the study, 1×10^5 cells were plated into 100 mm \times 20 mm round cell culture dishes in triplicate. One mL of medium was harvested from each dish at day 3. After harvesting, 1 mL of fresh medium was returned to the dish to keep the total volume stable. On day 4, all cells were trypsinized and counted. Cell counts ranged from 2.5×10^5 cells per plate (H3255) to 9×10^6 cells per plate (VAMT). All samples were sent for total protein quantitation immediately after collection.

Results

Analytical validation

The SMRP assay demonstrated an intra-assay CV of 2.3% at a mean of 4.6 nM, and 3% at a mean of 13.7 nM. The inter-assay CV was 3.5% and 3.7% at these same concentrations. The LOD was found to be 0.182 nM. For the OPN assay, precision was assessed at three levels and the following results were obtained: 5.8% at a mean of 1.9 ng/mL, 4.1% at a mean of 5.5 ng/mL, and 5.2% at a mean of 11.1 ng/mL. At these same levels, the inter-assay CV was found to be 8.5%, 8.4%, and 12.5%, respectively. The LOD for OPN was 0.032 ng/mL.

Reference interval assessment, ROC curves, and correlation analysis

We measured SMRP concentrations in 51 healthy volunteers and observed a range up to 0.82 nM. SMRP concentrations in blood samples from patients with mesothelioma and non-mesothelioma cancers are shown in Figure 1A. For OPN, we observed a range of 7.5–67.5 ng/mL in normal individuals. OPN concentrations in mesothelioma and non-mesothelioma patients are shown in Figure 1B. To determine the utility of these two markers in mesothelioma patients and normal volunteer samples, we designed ROC curves for each, shown in Figure 2A and B.

Interestingly, although each of the individual assays shows excellent correlation when replicates for the same assay are

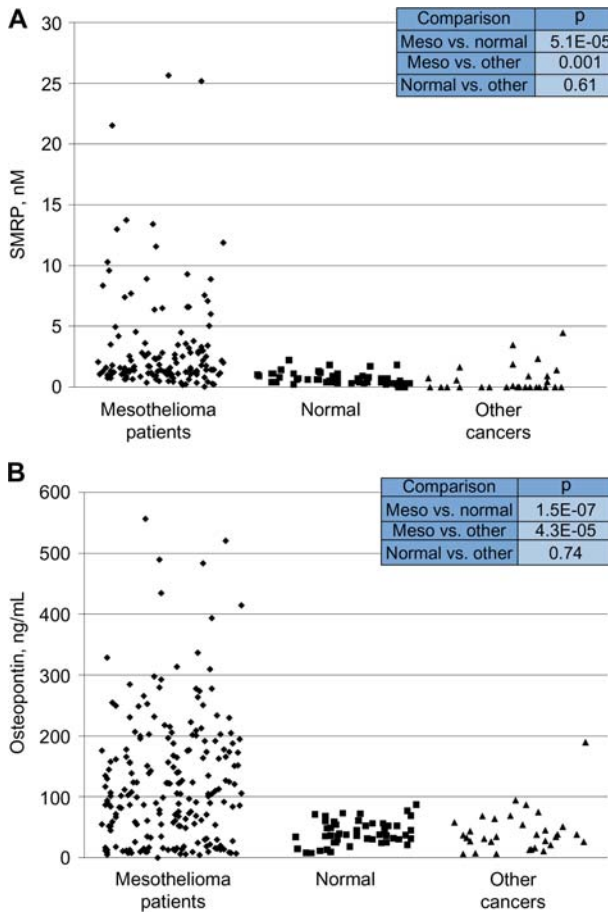


Figure 1 SMRP and OPN concentrations in patients and controls. (A) SMRP concentrations in mesothelioma (3.24 ± 4.27 , $n=139$), normal (0.72 ± 0.47 , $n=51$), and non-mesothelioma (other cancer) patients (0.63 ± 1.12 , $n=30$); (B) OPN concentrations in mesothelioma (129 ± 115 , $n=205$), normal (41 ± 19 , $n=51$), and non-mesothelioma (other cancer) patients (43 ± 35 , $n=30$). p-Values are indicated in inset for each of the comparisons.

compared, the concordance between mesothelin and OPN proteins in these same specimens is poor (Figure 3).

Tissue survey mRNA

We interrogated the GeoProfiles database for expression levels of mesothelin (MSLN, isoform 1) and OPN (isoform 1) mRNAs in different human tissues. MSLN was expressed highest in fetal lung, lung and trachea, with lower levels in several other tissues (Figure 4A). We also investigated OPN mRNA in the same sample set. OPN mRNA exhibited highest concentrations in kidney, with moderate expression in corpus, fetal brain, pancreas, and placenta (Figure 4B).

Protein concentrations in cell lines

We also tested corresponding cell line supernatants from eight representative cell lines. We found SMRP protein to be highest in Meso37 and HeLa cell lines and OPN to be highest in A549 and PC9 cell lines (Figure 5).

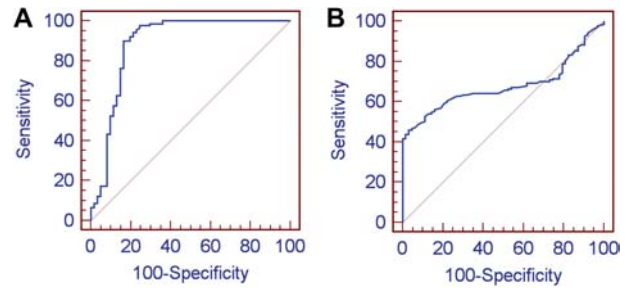


Figure 2 ROC curve analysis. Empirical ROC curve for separating mesothelioma patients vs. normal volunteers and patients with other cancers using (A) SMRP, and (B) OPN. The AUC (95% CI) values are: 0.89 (0.83–0.92) for SMRP and 0.68 (0.61–0.74) for OPN.

Pre-operative vs. post-operative blood concentrations from extrapleural pneumonectomy patients

We also obtained blood samples from seven consecutive patients with mesothelioma who underwent extrapleural pneumonectomy at Memorial Hospital. We measured blood concentrations of SMRP and OPN from samples obtained from these patients. The concentrations of SMRP decreased in five of seven post-surgical patient samples (Figure 6). For the remaining two patients, SMRP remained unchanged in one patient and increased slightly in the other. OPN did not show any consistent directional change in this small cohort (data not shown).

Discussion

The treatment of malignant pleural mesothelioma is controversial, but has evolved considerably during the past several decades. However, many areas need further improvement: easier and more accurate methods of pathological diagnosis, improved methods of staging and of selecting patients for surgery, assessment of response to chemotherapeutic and sur-

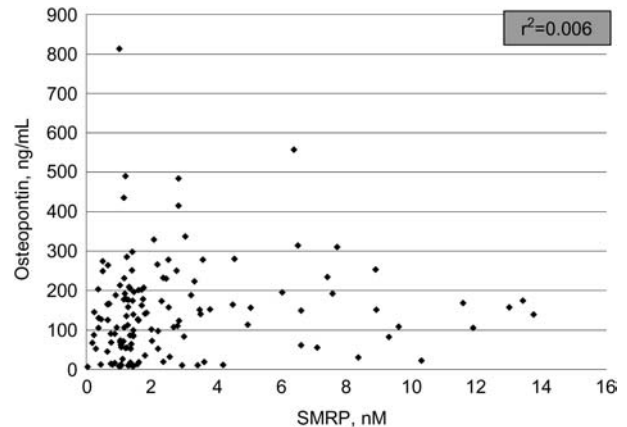


Figure 3 Correlation analysis: osteopontin vs. SMRP. Correlation was performed using 135 samples for which both mesothelin and osteopontin results were available.

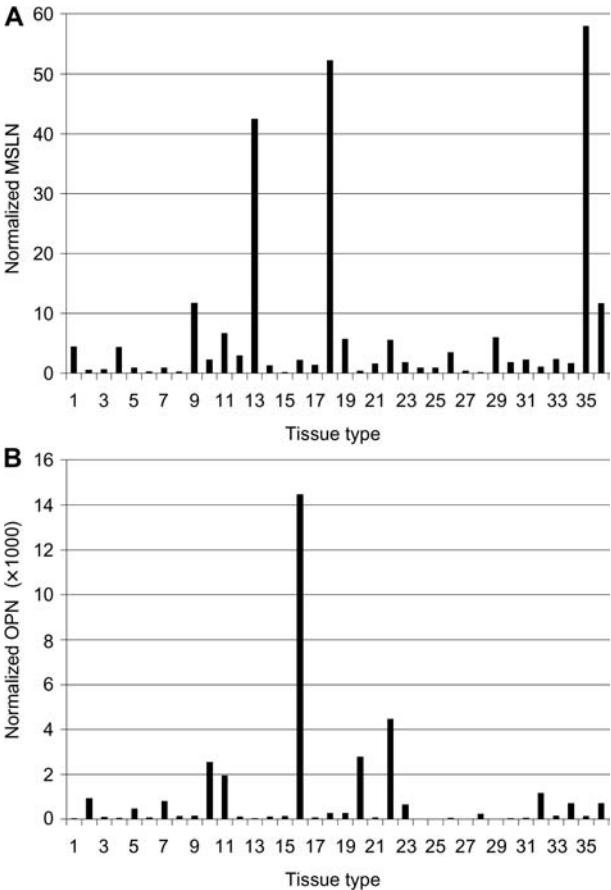


Figure 4 Concentrations of (A) MSLN and (B) OPN mRNAs in different human tissues. (1) Adrenal gland, (2) amygdala, (3) bladder, (4) bone marrow, (5) brain, (6) breast, (7) caudate nucleus, (8) cerebellum, (9) colon, (10) corpus, (11) fetal brain, (12) fetal liver, (13) fetal lung, (14) heart, (15) hippocampus, (16) kidney, (17) liver, (18) lung, (19) ovary, (20) pancreas, (21) pituitary gland, (22) placenta, (23) prostate, (24) smooth muscle, (25) salivary gland, (26) skin, (27) small intestine, (28) spinal cord, (29) spleen, (30) stomach, (31) testis, (32) thalamus, (33) thymus, (34) thyroid, (35) trachea, (36) uterus. Note that mRNA levels are normalized to GAPDH.

gical treatment, and assessment of disease recurrence in routine follow-up (28–33). Non-invasively obtained biomarkers specific to mesothelioma would improve care for all aspects of disease assessment and treatment (18, 20). In this study, we further characterized two markers, mesothelin and OPN, at the mRNA and protein levels, and defined the performance characteristics for ELISA methods for quantitative analysis in cell lines and blood.

Mesothelin is a differentiation antigen previously identified in pleura, peritoneum, and pericardium (34). In humans, it is encoded by the *MSLN* gene which produces a protein of 71 kDa. This preprotein is the target of certain furin-like proteases that cleave the molecule into two fragments. A 31-kDa amino terminal fragment called MPF is released into the circulation, and a 40-kDa product, mesothelin, remains membrane bound. Three molecular variants of mesothelin

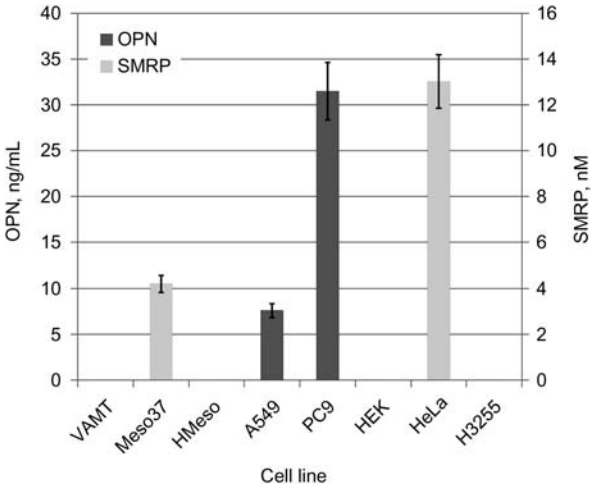


Figure 5 Concentrations of SMRP and osteopontin protein in cell line supernatants.

have been reported in the literature (accession numbers NM_005823.4, NM_013404.3, and AF180951), but only the existence of variants 1 and 3 has been demonstrated in ovarian and pancreatic cell lines and ovarian cancer tissue (17, 34).

OPN is a glycoprotein that is overexpressed in many human cancers including lung, breast, colorectal, gastric, ovarian, and melanoma (19). One important role for OPN lies in its ability to distinguish mesothelioma from asbestos exposure without malignancy (19, 25). There are three genes encoding isoforms a, b, and c of OPN (accession numbers NM_001040058.1, NM_000582.2, and NM_001040060.1) (26).

We validated assays for both SMRP and OPN, and used them for quantitation of the concentrations of their respective proteins in blood samples from patients with and without mesothelioma (Figure 1). Performance characteristics including intra-assay and inter-assay precision, LOD, reference

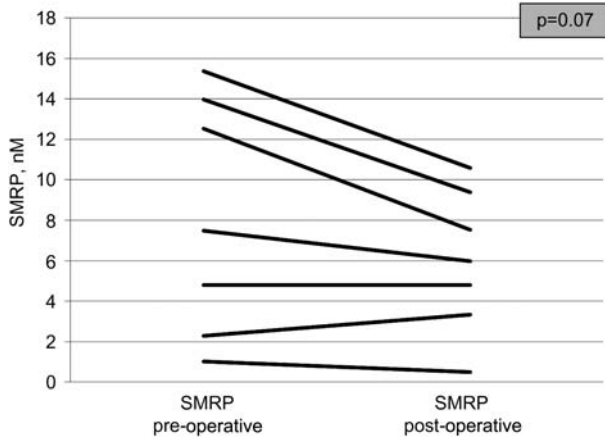


Figure 6 Comparison of SMRP concentrations in pre- and post-specimens from 7 extrapleural pneumonectomy (EPP) surgery patients.

interval assessment and comparison of concentrations of both proteins in mesothelioma and non-mesothelioma patients were determined. Our data suggest satisfactory performance of these assays in measurement of their respective analytes. They also provide baseline measurements and lay the groundwork for additional studies using blood samples from patients with and without mesothelioma and those undergoing treatment. Our results from LOD and precision studies are in close agreement with those of Beyer et al. (35). In addition, our reference interval data are narrower but consistent with the broader range established in this former study.

SMRP and OPN values do not correlate with each other (Figure 3). This suggests that at a functional level, the proteins are not redundant and thus likely to be involved in distinct pathways. This possibility is further reinforced by the different expression patterns of these two biomarkers. An *in silico* analysis of MSLN and OPN mRNAs in 36 human tissues was performed using GeoProfiles (<http://www.ncbi.nlm.nih.gov/sites/entrez>); see Figure 4. MSLN, the gene which encodes mesothelin, and whose protein product is detected by the SMRP assay, shows highest concentrations in fetal lung, lung, and trachea, with lower concentrations in several other tissues. In contrast, the *OPN* gene is expressed in highest amounts in kidney, with moderate levels in corpus, fetal brain, pancreas and placenta. The distinct expression pattern of these two molecules suggests different functions.

We measured the concentrations of both SMRP and OPN proteins in eight representative cancer cell lines. For SMRP, we observed highest protein concentrations in the supernatants of Meso37 and HeLa, with values below detection for the rest of the cell lines. It is remarkable that only one of the mesothelioma cell lines (Meso37) showed measurable concentrations of this marker. In reviewing microarray expression data obtained from analysis of these three cell lines, we noted that the Meso37 cell line expresses mesothelin mRNA at two orders of magnitude higher than VAMT and HMeso cell lines (data not shown). Thus, it is possible, and consistent with our ELISA results, that Meso37 produces mesothelin protein at concentrations that fall within the dynamic range of our assay and that the other cell lines which contain 100× less mesothelin mRNA produce values below detectable limits when measured with our assay.

For OPN, we observed highest concentrations of this protein in A549 and PC9 cell lines, with concentrations below the detection limit for the remaining cell lines. Both A549 and PC9 are derived from lung cancer patients. It is interesting that H3255, which is also a lung cancer derived cell line, does not show measurable concentrations of OPN, particularly since both PC9 and H3255 are sensitive to EGFR inhibitors. At the molecular level, PC9 cells contain EGFR with exon 19 deletion, and H3255 cells contain EGFR with L858R mutation. It is conceivable that such a difference could activate different downstream effector proteins with only PC9 cells activating a signal transduction pathway that results in mesothelin protein production and/or secretion. Interestingly, A549 cells which contain an activating KRAS

mutation, activates downstream signaling resulting in mesothelin secretion similar to PC9 cells.

To assess reference intervals, we measured the concentrations of SMRP and OPN in a cohort comprised of normal healthy volunteers, mesothelioma and non-mesothelioma (i.e., other cancer) patients. In 51 normal healthy volunteers, we observed a range of values up to 0.82 nM. This measured range is narrower, but falls within the reference interval stated in the product insert and based on the study by Beyer et al., who calculated the 99th percentile of the reference group to be 1.5 nM (35). This discrepancy can be reconciled by the size of our reference group ($n=51$) which is much smaller than that of theirs ($n=409$). When we measured samples from mesothelioma patients, they showed a broad range of results with maximum at 26 nM. Patients with prostate, breast, and ovarian cancer exhibited values up to 5 nM.

OPN concentrations were measured in the same cohort, and are shown in Figure 1B. Similar to SMRP, highest values were observed in mesothelioma patients (maximum of 525 ng/mL), lower concentrations in patients with other cancer (maximum of 200 ng/mL), and values levels in normal healthy volunteers (maximum of 70 ng/mL). Our observed values are somewhat lower than those suggested by the manufacturer (53–195 ng/mL), but this difference may be reconciled by the small cohort of 51 patients analyzed in our study, or the possibility that our patient population is different from that previously tested.

Finally, we analyzed SMRP and OPN concentrations in a small group of seven consecutive patients undergoing extrapleural pneumonectomy. We observed that SMRP, but not OPN, showed a decrement in five of seven post-surgical samples as compared to paired pre-surgical specimens. Although this result did not achieve statistical significance ($p=0.07$), it would be of great interest to assess concentrations of this marker at different time points and/or in a larger cohort of paired samples.

In conclusion, our data establish the reliability and reproducibility of two assays to quantitate SMRP and OPN proteins. We assessed reference intervals for both markers and show that the two do not correlate well in our sample set. Tissue expression surveys at the mRNA level show distinct profiles, as do the concentrations of the corresponding proteins in cell line supernatants. Finally, concentrations of SMRP but not OPN, decrease in post-surgical samples after extrapleural pneumonectomy. This is a promising preliminary result and warrants further investigation in a larger cohort.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

Research funding: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

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